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13. ABSTRACT *(Maximum 200 words)*

We examine the hypothesis that alterations in the regulation of growth by prolactin (PRL) and cAMP during mammary tumor progression are related to the ERK and JNK MAP kinase signaling pathways known to be affected by cAMP and pertussis toxin (PT)-sensitive G proteins. Mammary epithelial cells from normal mouse mammary glands were compared to pregnancy-dependent (PDT) and ovarian-independent (OIT) moue mammary tumors in serum-free, collagen gel cell culture. Inhibition of the ERK pathway by the MEK inhibitor, PD 098059, showed that this signaling pathway is involved in PRL-regulated proliferation of normal mammary epithelial cells and PDT. cAMP-stimulated proliferation of normal cells is also inhibited by PD as is the "autonomous" growth of OIT cells which occurs in the absence of added mitogens. However, cAMP inhibited ERK activity in normal cells and did not affect ERK activity in OIT or PDT indicating a lack of correspondence with cAMP stimulation (normal, PDT) and inhibition of growth (OIT). PRL was able to stimulate PT-sensitive ERK activity in normal cells only. JNK activity was not affected by cAMP or PT in normal cells and PDT. However, cAMP inhibited JNK activity in OIT suggesting a possible relationship to growth inhibition. These results suggest that the ERK cascade is only permissive for proliferation, and cAMP and hormones stimulate proliferation via other PT-sensitive pathways. The JNK pathway remains a candidate for a cAMP-sensitive proliferation associated pathway in OIT.

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Walter Dingman 7/1/98

PI - Signature Date

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Introduction.

Changes in signal transduction networks occur during mammary tumor progression that affect the proliferative response of the cells to exogenous factors. These alterations may be related to the loss of hormonal regulation of breast cancer which has a major impact on prognosis and therapy of breast cancer. The purpose of this research is to identify the mechanisms underlying the change in the proliferative response to 3'-5' cyclic adenosinemonophosphate (cAMP) that we have previously observed during mammary tumor progression in our rodent systems (1). Cyclic AMP is a potent mitogen for normal mammary gland epithelium. When compared to mammary tumors, however, difference in the proliferative response to cAMP were observed. Key findings were: *i*) a relative decrease in cAMP's growth stimulatory effect in hormone-dependent mammary tumors compared to normal mammary epithelium, *ii*) a switch to growth inhibition by cAMP in hormone-independent tumors, and *iii*) the inhibition of cAMP-stimulated proliferation by pertussis toxin (PT) in normal mammary epithelium. Neither the inhibitory effect of cAMP nor basal proliferation of hormone-independent mammary tumors was affected by PT (2). PT is a bacterial toxin that ADP-ribosylates the $G_{\alpha i}$ subunits of receptor-coupled heterotrimeric $G_{\alpha i \beta \gamma}$ proteins. This biochemical modification blocks activation of $G_{\alpha i \beta \gamma}$ by ligand occupied receptors resulting in the inhibition of signaling pathways activated by this class of G proteins. These findings indicate that through postreceptor crosstalk, pertussis toxin-sensitive $G_{\alpha i \beta \gamma}$ pathways modulate cAMP-mediated proliferation.

These preliminary results led to the hypothesis that a critical alteration in growth regulation related to signaling pathways affected by cAMP and pertussis toxin-sensitive G proteins occurs during transformation and progression of mammary tumors from hormone-dependent to hormone-independent growth. Furthermore, these effector pathways could play a pivotal role in coordinating multiple growth-stimulatory pathways and can modulate the hormonal responsiveness of mammary epithelium. An *in vitro* approach has been followed, taking advantage of our serum-free, primary cell culture system, to examine intracellular kinase pathways that may be altered during progression from normal mammary epithelium to hormone-independent mammary tumors.

The objective of these studies is to determine the mechanism underlying the change in proliferative response to cAMP between normal mammary epithelium and hormone-dependent mammary tumors (stimulation by cAMP) and hormone-independent mammary tumors (inhibition by cAMP). We have begun to examine the regulation by mammogenic hormones and cAMP of key mitogenic pathways that may be activated by $G\alpha i$ and cAMP, the ERK and c-jun (JNK) MAP kinase pathways (Specific Aim 1). The importance of the ERK pathway for mitogenesis is being assessed by the use of a specific inhibitor (PD 098059) of this pathway. For these studies, mammary epithelial cells from normal mouse mammary gland are being compared to pregnancy-dependent and ovarian-independent mammary tumors in serum-free cell culture to which hormones or cAMP are be added.

Materials and Methods

Reagents. Cell culture: Ham's F-12, Medium 199, and Dulbecco's Modified Eagle's medium (DMEM) were from GIBCO/BRL (Grand Island, NY); collagenase (CLS Type 2) was from Worthington Biochemical Co. (Freehold, NJ), Percoll was from Pharmacia Biotech (Piscataway,

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NJ). Rat tail collagen, solubilized in acetic acid, was prepared as described previously (3). Antibodies: ERK 1 (C-16), ERK 2 (C14), and jun kinase (JNK) were from Santa Cruz Biotechnology (Santa Cruz, CA); phospho-specific p44/42 (ERK 1 and ERK 2) antibody was from Promega (Madison, WI); phospho-tyrosine antibody (PY20) was from Transduction Laboratories (Lexington, KY). Biochemicals: MEK1 inhibitor PD 098059 was from Calbiochem (San Diego, CA) and pertussis toxin from Sigma Chemical Co (St. Louis, MO). MAP kinase substrate peptide containing the MAP kinase consensus phosphorylation sequence (amino acids 95-98 of MBP) was from Santa Cruz Biotechnology. [(γ^{32}P]-ATP was from DuPont-NEN. Gst-c-jun fusion protein substrate was prepared using a fusion construct provided by Roger Davis. EGF was from Collaborative Research (Waltham, MA) and Protein A and G agarose was from Sigma Chemical Co (St. Louis, MO).

Cell culture and tissues. Pregnancy-dependent mammary tumors were propagated *in vivo* by intrafat pad transplants in estrogen and progesterone (injection or pellet) DDD mice. Ovarian-independent mammary tumors were raised by subcutaneous transplantation of tumor pieces in virgin DDD mice. These hormone-independent tumors grow rapidly in virgin hosts. Normal tissues were from mature virgin Balb/cAnNCrlBR mice obtained from Charles River.
Normal and tumor tissues were dissociated with collagenase (0.1%) and purified epithelial cells obtained by Percoll gradient centrifugation as described previously (3). For growth experiments, cell organoids were cultured for 10 days within collagen gels as described (3). The basal medium used for cell growth was composed of a 1:1 (v:v) mixture of Ham's F-12 and DMEM buffered with 20 mM HEPES and 0.67 g/l sodium bicarbonate, and supplemented with 10 µg/ml insulin, 100 U/ml soybean trypsin inhibitor, 1 µg/ml α-tocopherol succinate and other additives as indicated. When PD 098059 (in DMSO) was added, it was mixed well by vortexing in fresh medium at the time of medium changes. Cell number was determined by fluorometric DNA assay using diaminobenzoic acid (4) and standard curves using diploid tumor cells. Cell cultures for kinase assays were established as as above using 10 or 6 cm culture dishes with 5 to 10 million cells per culture. Cells were cultured for 4-6 days in serum-free medium (above) prior to the addition of test factors (cAMP, Prolactin (PRL), progesterone (P), EGF). When cAMP was added in combination with EGF, it was added 1 hr. prior to the addition of EGF. Pertussis toxin (100 ng/ml) was added overnight (to allow activation by the cells) prior to the addition of test factors.

Preparation of Cell Extracts. After incubation for various times, cultures were terminated by aspiration of the culture medium followed by blotting of the gels on filter paper and transfer of the dehydrated gels to 0.5 ml of lysis buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% (v/v) Triton X-100, 40 mM β-glycerophosphate, 40 mM PNPP, 200 µM sodium orthovanadate, 100 µM phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml leupeptin, 1 µg/ml pepstatin A, 1 µg/ml aprotinin. The lysates were mixed by vortexing and left on ice for 60 min. before centrifugation (10 min, 13,000 x g, 4^{o} C). Supernatants were used for immunoprecipitations and western blot analysis. Protein concentration was determined using the BCA assay by Bradford.

ERK Activity Assay. MAPK assays were performed using an immune complex kinase filter assay as described previously with minor modifications (5). Briefly, lysate protein (0.3 mg) was immunoprecipitated (>90% efficiency) with Protein A-sepharose conjugated with 3 μg anti-MAP kinase rabbit polyclonal antibodies (ERK1 and ERK2). The Protein A was then resuspended in kinase reaction buffer containing 20 μM ATP, 0.25 mg/ml myelin basic protein peptide, and 10 μCi of [γ^{32}P]-ATP. After incubation, samples were centrifuged and aliquots spotted on p81 cation-exchange filter papers (Whatman)which were washed in with phosphoric acid and radioactivity was quantitated by liquid scintillation counting.

Western Blotting for ERKs. Sample lysates containing 20 μg of total protein were electrophoresed on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes by electroblotting. Membranes were blocked with TBS buffer containing 5% (w/v) dry milk and 0.1% Tween and incubated with anti-phospho-ERK antibody (diluted at 1:3,000), ERK1 and ERK2 antibodies (diluted at 1: 10,000). For all immunoblotting, a horseradish peroxidase-conjugated secondary antibody was utilized to allow detection of the appropriate bands using enhanced chemiluminescence (Amersham Corp.). For data analysis, ERK1 and ERK2 bands were scanned using a Molecular Dynamics Personal Densitometer using ImageQuant software.

c-JUN Kinase (JNK) Assay. JNK activity was determined by kinase assay of JNK 1 immunoprecipitates as described by Dhanwada et al.(6). Briefly, JNK 1 was immunoprecipitated from cell lysates (0.15 mg protein) with JNK 1 antisera conjugated to Protein G beads. Beads were then washed and incubated for 15 min in kinase buffer containing 10 μCi of [γ^{32}P]-ATP, and 8 μg of c-jun fusion protein substrate. The reaction was stopped by the addition of 2X sample buffer and the samples electrophoresed on 10% SDS PAGE gels. The gels were then stained with commassie blue, dried and exposed to x-ray film. Densitometry was performed on the substrate fusion protein band as described for ERKs above.

Results and Discussion

The studies described below were to be carried out in the first year of the the grant as outlined in the Statement of Work. Our aims were to determine the effect of PD 098059 on hormone and cAMP-stimulated proliferation and on the activity of the ERK and c-Jun kinase cascades. Most of the aims have been completed.

1. Role of the ERK pathway in mitogenesis stimulated by hormones and cAMP.
The goal of these studies was to determine if hormone- and cAMP-stimulated proliferation of normal and tumor mammary epithelial cells is dependent on the activation of the MAP kinase pathway. PD098059 (PD) is a highly specific inhibitor of the ERK cascade (7). It inhibits *raf* activation of MEK, the upstream kinase that phosphorylates and activates ERKs 1, 2 (referred to as ERKs). This inhibitor has been used to evaluate the role of this kinase cascade in the proliferative response to

mitogens. We evaluated the effect of PD on cell proliferation stimulated by hormones (P and/or PRL), cAMP, and EGF in mammary epithelial cells from normal virgin mice and from pregnancy-dependent (PDT) and ovarian-independent (OIT) mammary tumors. **Fig 1** (p. 12, Appendix) is a representative experiment showing that PD inhibited proliferation stimulated by P + PRL (although the response is small in this experiment) in normal cells. In PDT cells, which are very mitogenically responsive to PRL, PD inhibited PRL-stimulated growth (**Fig 2**). PD inhibited cAMP-stimulated growth in normal cells (**Fig 1**) while the low or negligible response to cAMP-alone in PDT made it difficult to evaluate this effect. For comparison, EGF stimulated proliferation for both tissues, is inhibited in a dose-dependent manner by PD. OIT grows in basal medium and when PD is added to this medium we observed growth inhibition which was complete at the highest concentration tested (**Fig 3**). This indicates that growth of the OIT is dependent to a large extent on a functioning ERK cascade. As found previously, since cAMP inhibits the growth of this tumor (**Fig 5**) we hypothesized that it might be inhibiting ERK activation via crosstalk as described for nonepithelial cell types. This possibility was evaluated in ERK activity assays (below).

We have found previously that pertussis toxin (PT) inhibits cAMP, and P+PRL-stimulated proliferation in normal mammary epithelium and we now find that it also inhibits PRL-stimulated growth in PDT (**Fig 4**). We could not determine its effect on cAMP-stimulated growth in PDT since the cAMP effect on proliferation was minimal. In contrast, the growth of OITs is not affected by PT and cAMP is growth inhibitory (**Fig 5**).

In summary, hormone-stimulated proliferation in normal mammary epithelium and PDT is partially dependent upon ERK activation. In addition, PT sensitivity of the hormonal response indicates that a PT-sensitive G protein activated pathway is also utilized. For normal mammary epithelium, cAMP mitogenesis is dependent in part on the ERK pathway. The ERK pathway is required for OIT proliferation, which is dependent only upon insulin supplementation of the serum-free medium.

2.Effect of hormones and cAMP on the activation of ERKs and JNK in cultured mammary epithelial cells.

PT-sensitive $G\alpha i\beta\gamma$ coupled receptors can activate the ERK pathway (8-10) via $G\alpha i\beta\gamma$ activation of ras-dependent (11, 12) and ras-independent mechanisms depending on the cell type (13, 14). In cells where cAMP inhibits proliferation it also inhibits the activation of the MAP kinase pathway, possibly by blocking raf activation (15-18). However, a recent report (19) indicated that cAMP mediated growth inhibition in the CCL39 fibroblast line is not due to inhibition of ERK kinase but to some other event. In cells where cAMP is stimulatory to growth, cAMP stimulates ERK activity (20).

Another MAP kinase pathway associated with the control of proliferation control can be affected by G protein coupled receptors is the JNK/SAPK pathway (21-24). Observation of cAMP/PKA inhibition of the JNK pathway in lymphocytes (25 , 26) now offers another possible mechanism for cAMP inhibition of growth if JNK can be shown to activated and associated with proliferation in mammary epithelium. Thus, we can see how differential activation of these pathways may be an important regulatory alteration occurring during tumor progression.

To determine if hormones and cAMP stimulated ERK and JNK activity, mammary tissues from normal mice, and PDT and OIT tumors from were cultured in serum-free collagen gel culture and lysates prepared for analysis of kinase activities. Initial experiments examining the activation of ERK activity by EGF utilized culture on collagen-coated plates in order to rapidly lyse the cells at the termination of incubations. These experiments focused primarily on EGF which markedly activates ERKs. Subsequent experiments examining hormonal and cAMP effects on kinase activity used cultures within collagen gels to match the conditions for the growth experiments. EGF activation of ERKs is similar under both culture conditions.

a) Normal mammary epithelium.

The proliferation of normal mammary epithelium is stimulated by cAMP but we have found that cAMP does not activate ERKs in these cells. Time course studies (**Fig 6**) have shown that, in fact, it may slightly inhibit ERK activity. ERK activity, however, is inhibited by PT (**Figs 7,8**) in basal medium or in the presence of cAMP. Thus, at least when cAMP is administered alone, activation of the protein kinase A pathway does not appear to positively modulate the ERK pathway. These results were somewhat surprising since a reasonable expectation was that cAMP would have a positive effect on ERK activity associated with the proliferative response. PRL appears to have a small stimulatory effect on ERK activity which is inhibited by PT (**Fig 8**). This effect of PRL is variable in that consistent time courses of activation have been difficult to achieve. We believe that PRL does not strongly activate this pathway especially in comparison to EGF. PT inhibition of PRL's stimulation of ERK activity (previously shown for PRL by others) was not unexpected for this hormone.

JNK activity was not affected by cAMP in normal epithelium (**Fig 9**). We find that there can be variation in the activity of cells cultured in basal medium over time which may reflect the sensitivity of this kinase to external physical factors incurred during handling of the cultures during the experiments. This complicates our analysis but **Fig 9** is representative of 3 experiments and shows that we cannot reproducibly observe a stimulatory or inhibitory effect of cAMP on JNK. In contrast, we observe that EGF can reproducibly stimulate JNK activity in these cells (not shown) indicating to us that a strong positive response (>2X) is detectable under our assay conditions. Unlike for ERKs, PT had no effect on JNK activity in basal medium or in the presence of cAMP (**Fig. 10**).

b) Pregnancy-dependent mammary tumors (PDT).

Multifold enhancement of proliferation of PDT is induced by EGF and by PRL, whose effect is potentiated when it is combined with P (P+PRL). Similar to normal mammary tissue, EGF is a strong activator of ERKs but in extensive experiments P+PRL had no effect of ERK activity (**Fig 11**). This response was checked in time course experiments of up to 6 days over a time when proliferation is maximal in collagen gels. Thus, contrary to normal cells, PRL does utilize the ERK pathway. Its effect on the JNK pathway has yet to be examined. cAMP which alone is not or marginally stimulatory to proliferation had no effect on either ERK (**Fig 12**) or JNK (**Fig 13**) activity. Neither did PT effect ERK or JNK activity when added alone to basal medium or, for

ERKs, also in the presence of EGF. These results indicate that these two kinase cascades are activated by EGF but not by hormones and cAMP in PDT. This result then leads us to consider that while the ERK pathway is important for mitogenesis initiated by these factors (as shown by PD inhibition), it serves a permissive role. The nature of this role is undefined but can be related to matrix interactions and possible co-regulation of transcription factor activity downstream of ERKs and JNKs.

c) Ovarian-independent mammary tumors OIT).

These tumors proliferate in serum-free medium with only insulin supplementation and their proliferation is not affected by the addition of hormone and growth factors but it is inhibited by cAMP, and agents that can raise intracellular cAMP levels such as epinephrine, prostaglandin E_2, and forskolin. Because cAMP is growth inhibitory and growth of OITs is inhibited by PD, we evaluated the effect of cAMP on ERK activity. **Fig 14** is a summary of 3 experiments showing that cAMP does not significantly affect ERK activity. We see no consistent inhibition of ERK which we might expect if cAMP inhibition of growth is mediated via this pathway. Thus, no crosstalk between cAMP/PKA and the ERK pathway affecting the control of ERK activity is observable. It is also interesting to note that the basal level of activated or phosphorylated ERK appears to be lower in OITs relative to normal and PDTs. This is based on immunoblotting with phospho-specific ERK antibodies and needs to be confirmed with activity assays. Epinephrine and prostaglandin E_2 are ligands to G protein-coupled receptors and may signal via pathways other than the cAMP pathway. Preliminary experiments examining the effect of epinephrine and prostaglandin E_2 on ERK activity indicate that these factors can dramatically inhibit ERKactivity suggesting that these factors activate non-cAMP signaling pathways that affect the ERK pathway.

In contrast, although fluctuation in basal levels is present, we observe that cAMP tends to inhibit JNK activity. **Fig 15** shows three separate time course experiments illustrating this effect (data for each is normalized to Basal 4 minutes. The stimulatory response at 4 min (Tumor 2) has been observed for this and another OIT but is not consistently present. More experiments are needed to confirm this inhibitory response but these results suggest that cAMP differentially affects the ERK and JNK pathways in these tumor cells and may play a role in the negative effect of cAMP on proliferation.

Summary and Conclusions

The participation of the ERK pathways in proliferation stimulated by hormones, EGF, and cAMP was determined in primary, serum-free culture experiments using normal and tumor tissues varying in their mitogenic response to these factors. Use of the MEK inhibitor, PD90859, showed that the ERK pathway is at least permissive for the mitogenic response to hormones and cAMP for normal mammary epithelium and PDT epithelium. The "autonomous" growth of OITs was also dependent upon the ERK pathway.

We have established assays to assess the activity of ERK 1,2 and c-jun kinase. In addition, immunoblotting with phospho-specific antibodies to ERKs has allowed us to use this technique

(which mirrors the results of kinase assays) to monitor ERK activation. When the effect of hormones and cAMP on ERK activity was assessed, hormonal stimulation of activity was only observed for normal mammary epithelium with no effect observed for the PDT. Thus, the strong mitogenic response to PRL in PDT is not dependent on the activation of the ERK cascade. cAMP had no effect on ERK activity in either PDT or OIT but contrary to expectation, inhibited ERK activity in normal mammary epithelium in spite of its mitogenic effect. In the OIT where cAMP is growth inhibitory, cAMP had no effect on ERK activation. These results suggest that although the ERK pathway is required for growth, a specific role for it in transducing the mitogenic response (stimulatory or inhibitory) to hormones or cAMP is not apparent. This interpretation does not exclude an important role for the ERK pathway since inhibition of this pathway does inhibit hormone and cAMP-stimulated proliferation and there can be crosstalk at points downstream of kinase activation. Rather they suggest that non ERK signaling pathways induced by ligand binding or protein kinase A activation are involved in the hormonal and cAMP initiation of the mitogenic response. Examination of the JNK pathway showed that while EGF stimulates JNK activity in normal and PDT mammary epithelium, hormones and cAMP have no effect. This pathway appears not to be activated in response to these factors and probably is not directly invoked as part of their mitogenic response. For the OITs, however, cAMP appears to be inhibitory to JNK activation raising the possibility that this pathway may be involved in cAMP's inhibitory effect on proliferation.

The role of $G\alpha i\beta\gamma$ in modulating the cAMP response appears not to occur through a modulation of ERK activity in normal or PDT mammary epithelium since these factors appear to activate a different signaling pathway(s). However, PRL activation of ERKs in normal mammary epithelium was inhibited by PT indicating that hormonal mitogenesis is partially dependent upon G protein pathways. In contrast, the effect of PT on hormonal stimulation of PDT proliferation appears not to involve modulation of the ERK cascade since hormonal stimulation does not significantly affect this signaling pathway. Thus, other PRL regulated pathways whose activation is affected by a PT-sensitive G protein are involved. We hope to identify this pathway by further exploration PRL receptor activated signaling pathways such as the JAK/STAT pathway and src family protein kinases.

For the next year we will, in addition to beginning the examination of known PRL activated pathways, we will monitor the effect of hormones, cAMP, and PT on the phosphorylation of transcription factors (such as Elk-1, crebs, ER, PR) that are substrates for MAP kinases and protein kinase A to detect sites of potential crosstalk of signaling pathways at this level. Studies on the involvement of insulin in signaling by hormones, cAMP, and PT and the examination of the activation of $G\alpha i\beta\gamma$ will be persued as described in our Statement of Work.

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Appendix

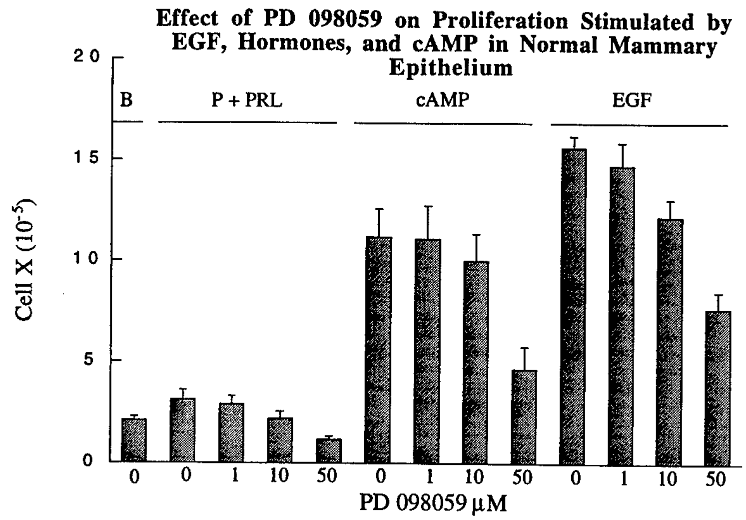


Figure 1

Cells were cultured in serum-free medium for 10 days before termination. Hormones (Progesterone (10^{-7}M) + Prl (1 ug/ml), EGF (10 ng/ml), or cAMP (100 ug/ml+IBMX (0.1mM)) were added in the absence and presence of PD. B is basal serum-free medium with no additives.

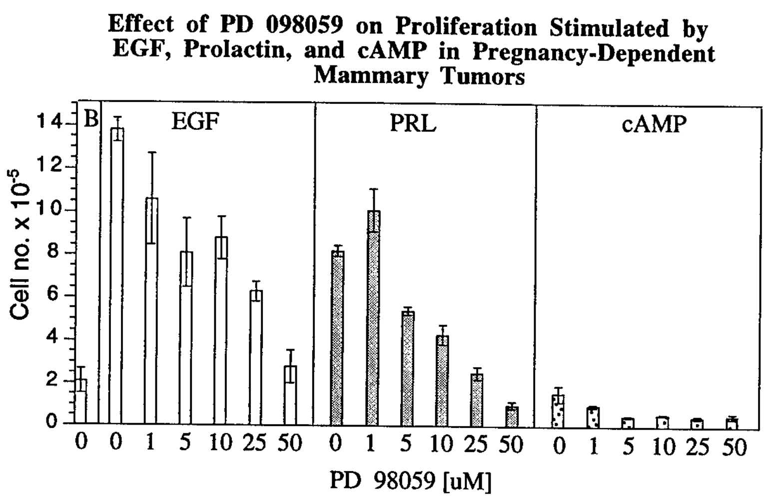


Figure 2

Cells were cultured for 10 days in serum-free medium containing EGF (10 ng/ml), Prolactin (PRL, 1 ug/ml), or cAMP (250 ug/ml) in the absence or presence of the MEK inhibitor PD 098059. B is basal medium control. Mean and sd of triplicate cultures is plotted.

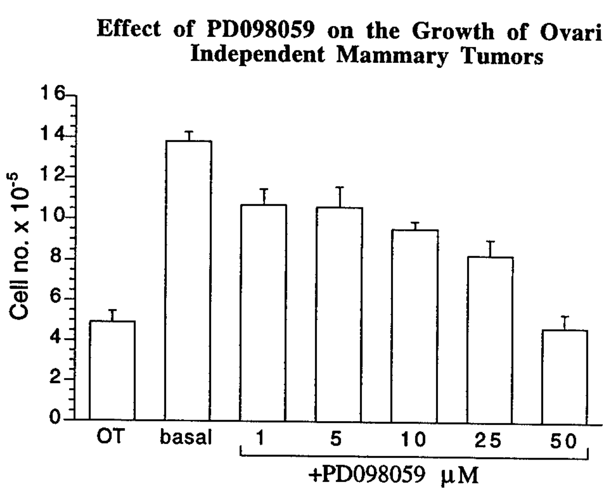


Figure 3

Cells from OIT were placed in serum-free, collagen gel culture +/- the MEK inhibitor PD098059. After 12 days cultures were terminated for DNA assay. OT is the starting cell number. Mean and sd of triplicate cultures.

Effec of Pertussis Toxin (PT) on Hormone-Stimulated Growth in Cultured Cells From Pregnancy-Dependent Tumors

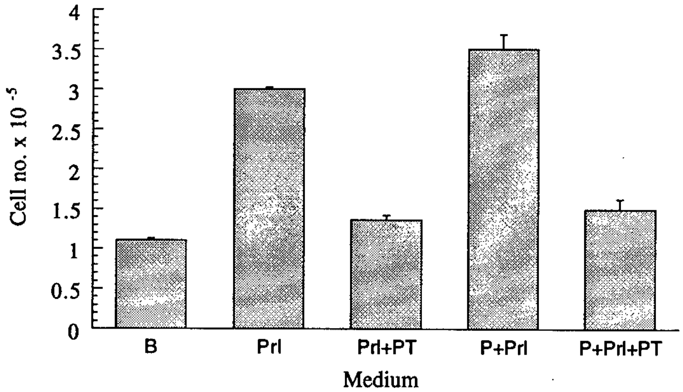


Figure 4

Cells were cultured for 10 days in serum-free medium with the above additives. Prolactin (Prl, 1ug/ml), Pertussis Toxin (PT 100 ng/ml), Progesterone (P, 10-7M). B is basal medium control. Mean and sd of triplicate cultures.

Growth of Ovarian-Independent Mammary Tumors in serum-free, collagen gel culture.

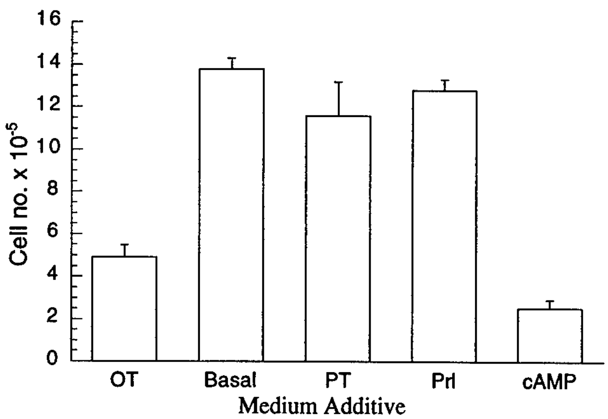
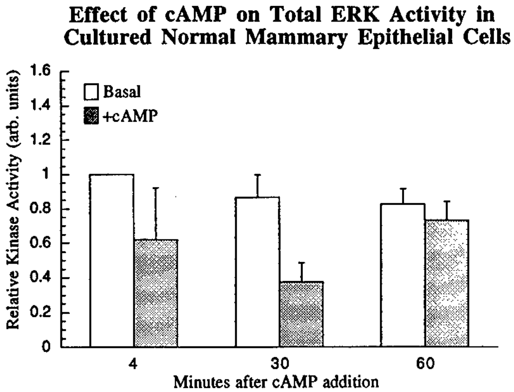


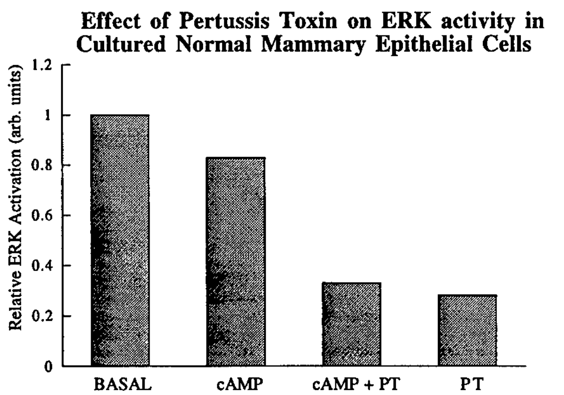
Figure 5

Cell number determined after 12 days culture in serum-free medium +/- Pertussis toxin (PT, 100 ng/ml), prolactin (Prl, 1 ug/ml), or db-Cyclic AMP (cAMP, 100 ug/ml).



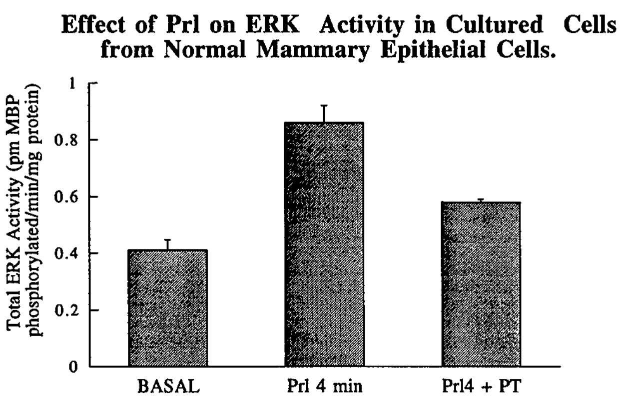
Cultures were terminated at the indicated times after cAMP (100 ug/ml) addition. Average of 2 experiments. Data are normalized to Basal 4 min.

Figure 6



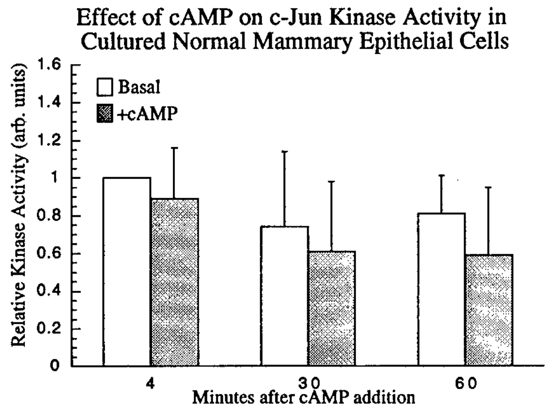
cAMP (100 ug/ml) was added for 4 min before termination. Activity determined by densitometry is normalized to basal. Cultures were exposed to pertussis toxin (PT, 100 ng/ml) overnight.

Figure 7



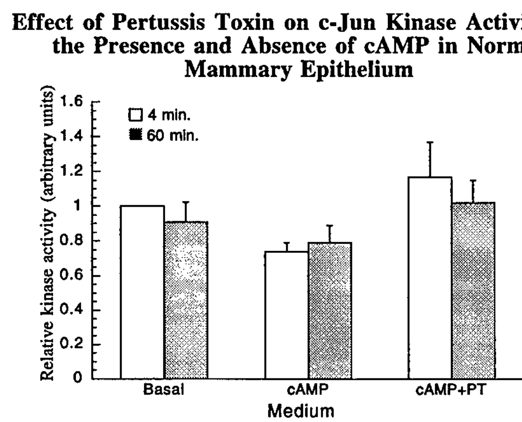
Prolactin (Prl, 1ug/ml))was added for 4 minutes prior to termination of control (Basal) and treated cultures. PT was added overnight

Figure 8



Cultures were terminated at the indicated times after cAMP (100 ug/ml) addition. Average of 3 experiments. Data are normalized to Basal 4 min.

Figure 9



Average of two experiments using cells from virgin mice.
PT (100 ng/ml) treatment was overnight. Basal control or +cAMP cultures were terminated 4 or 60 min after cAMP addition. Data are normalized to Basal 4 min.

Figure 10

Effect of Hormones and EGF on Total ERK Activity in Cultured Pregnancy-Dependent Mammary Tumor Cells

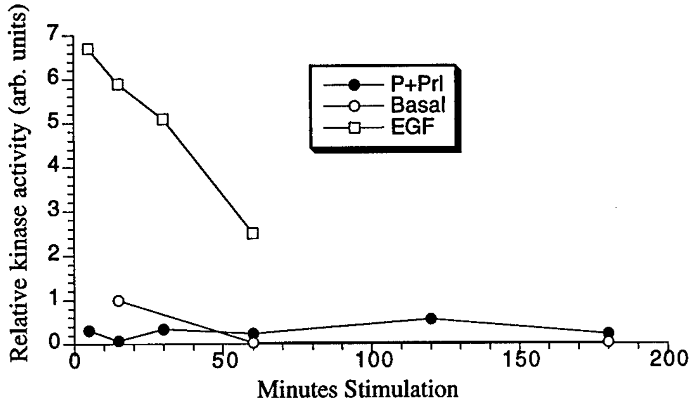


Figure 11

Cells were cultured in serum-free medium for 6 days then Prolactin (Prl, 1 ug/ml) + Progesterone (P 10-7M), or EGF (10 ng/ml) were added for the indicated times (5-180 minutes) prior to termination. Data are normalized to basal at 15 minutes.

Effect of cAMP and Pertussis Toxin on ERK Activity in Cultured Cells from Pregnancy-Dependent Mammary Tumors

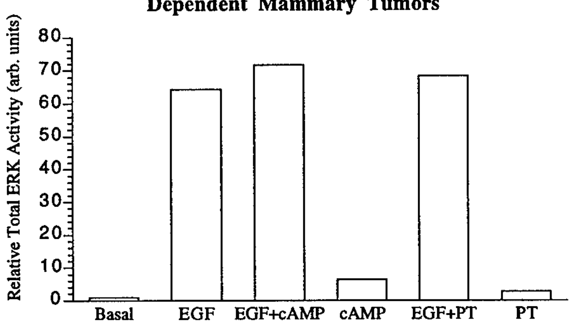


Figure 12

Cells were cultured in the absence (Basal) or presence of the indicated additives. EGF was added for the optimum time of 4 minutes in the absence and presence of cAMP (100 ug/ml) or pertussis toxin (100 ng/ml). cAMP was added for 60 minutes prior to EGF addition in EGF+cAMP. PT was added overnight. Data normalized to Basal.

Effect of cAMP and Pertussis Toxin on c-Jun Kinase Activity in Cultured Cells From Pregnancy-Dependent Tumors

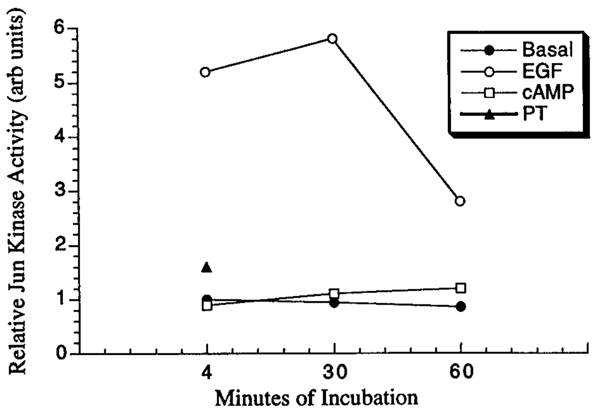


Figure 13

Cells were cultured for the indicated times in serum-free medium in the absence and presence of EGF and cAMP. PT was added overnight. Data normalized to Basal at 4 min.

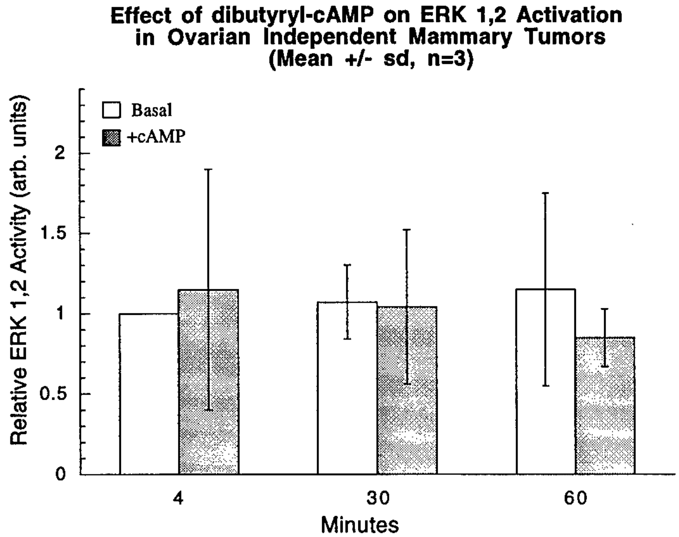


Figure 14

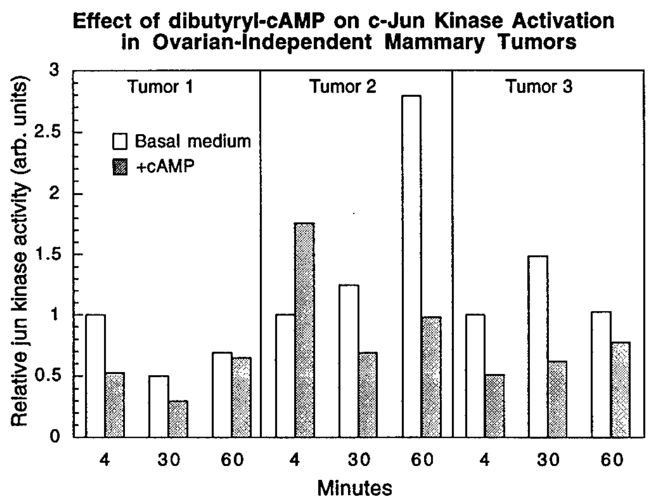


Figure 15